Stability and Cu(II) Binding of Prion Protein Variants Related to Inherited Human Prion Diseases

Grazia M. Cereghetti,* Arthur Schweiger,† Rudi Glockshuber,* and Sabine Van Doorslaer†

*Institute of Molecular Biology and Biophysics and †Department of Physical Chemistry, Swiss Federal Institute of Technology, Hönggerberg, CH-8093 Zurich, Switzerland

ABSTRACT All inherited forms of human prion diseases are linked with mutations in the prion protein (PrP) gene. Here we have investigated the stability and Cu(II) binding properties of three recombinant variants of murine full-length PrP(23–231)-containing destabilizing point mutations that are associated with human Gerstmann-Sträussler-Scheinker disease (F198S), Creutzfeld-Jakob disease (E200K), and fatal familial insomnia (D178N) by electron paramagnetic resonance and circular dichroism spectroscopy. Furthermore, we analyzed the variants H140S, H177S, and H187S of the isolated C-terminal domain of murine PrP, mPrP(121–231), to test a role of the histidine residues in Cu(II) binding. The F198S and E200K variants of PrP(23–231) differed in Cu(II) binding from the wild-type mPrP(23–231). However, circular dichroism spectroscopy indicated that the variants and the wild type did not undergo conformational changes in the presence of Cu(II). The D178N variant showed a high tendency to aggregate at pH 7.4 both with and without Cu(II). At lower pH values, it showed the same Cu(II) binding behavior as the wild type. The analysis allowed for a better location of the Cu(II) binding sites in the C-terminal part of the protein. Our present data indicate that hereditary forms of prion diseases cannot be rationalized on the basis of altered Cu(II) binding or mutation-induced protein destabilization alone.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) such as bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans are supposed to be caused by the scrapie form of prion protein (PrP^{Sc}), the abnormal, oligomeric form of the cellular prion protein (PrP^{C}) of the host. Both forms of prion protein (PrP) appear to have identical covalent structures but differ in tertiary structure and oligomerization state (Griffith, 1967; Prusiner, 1982, 1991).

PrP^{C} is a glycosyl phosphatidylinositol-anchored cell-surface glycoprotein that is strongly expressed in cells of the central nervous system (Kretzschmar et al., 1986; Stahl et al., 1987; Stahl et al., 1990). It consists of 209 amino acids and is covalently modified by the addition of several high-mannose glycans (Endo et al., 1989). In contrast, PrP^{Sc} is an insoluble, proteinase K-resistant oligomer of PrP that accumulates in the brain during infection with prions (Weissmann et al., 1996).

Evidently, full insight into prion propagation requires knowledge of the three-dimensional structures of PrP^{C} and PrP^{Sc} and the mechanism of formation of PrP^{Sc} from PrP^{C} (Jarrett and Lansbury, 1993; Prusiner, 1991). The determination of the NMR structure of recombinant PrP^{C} from several organisms including mice (Riek et al., 1996; Riek et al., 1997), hamsters (Donne et al., 1997), cattle (Lopez Garcia et al., 2000), and humans (Zahn et al., 2000) has shown that PrP^{C} consists of a well-structured C-terminal domain comprising residues 121–231 and a flexibly disordered N-terminal region (residues 23–120). The C-terminal domain PrP(121–231) contains three α-helices and a two-stranded, antiparallel β-sheet. The only two cysteine residues of the protein, Cys-179 and Cys-214, form a buried disulfide bridge connecting helices 2 and 3. Unfortunately, the tendency of PrP^{Sc} to form insoluble aggregates has so far prevented its structure determination at the atomic level. However, Fourier transform infrared (FTIR) spectroscopy showed that PrP^{Sc} has an increased β-sheet content compared to PrP^{C} (Pan et al., 1993).

More than 20 different mutations in the prion protein are known to be associated with inherited TSEs in humans (Collinge, 2001), i.e., the inherited form of CJD (Bell and Ironside, 1993; Spudich et al., 1995), the Gerstmann-Sträussler-Scheinker syndrome (GSS; Ghetti et al., 1995; Unverzagt et al., 1997), and fatal familial insomnia (FFI; Goldfarb et al., 1992; Gambetti et al., 1995). Interestingly, all disease-related point mutations in human PrP are located within segment 90–231, which forms the protease-resistant core of the PrP^{Sc} oligomer (Safar et al., 1990, Weissmann et al., 1996). Nevertheless, the mechanistic role of mutations in spontaneous formation of PrP^{Sc} in inherited TSEs is not evident. It is however quite evident that neither destabilization of PrP^{C} nor higher stability of PrP^{Sc} are general mechanisms underlying the spontaneous generation of prions in patients with inherited TSEs (Prusiner, 1997; Cohen et al., 1994; Huang et al., 1994; Liemann and Glockshuber, 1999; Swietnicki et al., 1998).

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Address reprint requests to Sabine Van Doorslaer, Laboratory for Spectroscopy in Biophysics and Catalysis, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium. Tel.: +31-03-8202461; Fax: +31-03-8202470; E-mail: sabine.vandoorslaer@ua.ac.be.

Grazia M. Cereghetti's present address is Biological Chemistry Department, Viale G. Colombo 3, 35121 Padova, Italy.

Sabine Van Doorslaer's present address is Laboratory for Spectroscopy in Biophysics and Catalysis, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium.

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A number of recent observations indicate that PrP may be a metalloprotein in vivo. It has been claimed that the levels of copper in the brains of prion-protein-knockout ($PrP^{0/0}$) mice are lower than in wild-type mice (Brown et al., 1997), although this finding has not been reproduced by other workers (Waggoner et al., 2000). Furthermore, PrP has also been proposed to function as a copper-transport protein (Pauly and Harris, 1998). In vitro studies have shown that synthetic peptides containing the octarepeat part of the N-terminal region of PrP (a fourfold repeat of eight amino acids with the consensus sequence PHGGGWGQ) bind 1–6 Cu(II) ions (Brown et al., 1997; Hornshaw, McDermott, and Candy, 1995; Hornshaw et al., 1995; Stöckel et al., 1998; Viles et al., 1999; Miura et al., 1999; Aronoff-Spencer et al., 2000; Cereghetti et al., 2001; Burns et al., 2002; Qin et al., 2002). Although Cu(II) binding was long thought to take place only in the octarepeat region of PrP^{C}, recent findings indicate that additional Cu(II) bindings are present in the more C-terminal segments. Fluorescence titration showed that recombinant human PrP(91–231) has a high-affinity Cu(II) binding site located in the region around His-96 and His-123 and weaker binding upstream of this region (Jackson et al., 2001). Cu(II) binding at pH 8 involving His-96 and His-111 was shown using x-ray absorption fine-structure spectrometry (Hasnain et al., 2001). In our earlier electron paramagnetic resonance (EPR) studies, we also showed that the structured C-terminal domain of murine PrP(23–231) is able to interact with Cu(II) ions (Cereghetti et al., 2001; Van Doorslaer et al., 2001). Three pH-dependent Cu(II)-coordination types are observed in the isolated mPrP(121–231) domain (Cereghetti et al., 2001). Binding of one Cu(II) ion to the C-terminal region of PrP at physiological concentrations was also found by Kramer et al. (2001).

To investigate a possible relationship between metal binding and disease, we analyzed by EPR spectroscopy the binding of Cu(II) to three recombinant mPrP(23–231) variants that correspond to the three different phenotypes of inherited human prion diseases (Fig. 1 *a*): D178N (with M129V, FFI), F198S (GSS), and E200K (inherited CJD). We find that two of the mPrP(23–231) variants partially show anomalous Cu(II) binding behavior compared to the wild type. Furthermore, equilibrium unfolding transitions of these variants of full-length PrP(23–231) showed different degrees of destabilization. Additionally, the recombinant histidine replacement variants H140S, H177S, and H187S of mPrP(121–231) were investigated to identify the histidine-related Cu(II) binding sites (Fig. 1 *a*).

MATERIALS AND METHODS

Plasmids and oligonucleotides used for site-directed mutagenis

The mPrP(23–231) variants D178N, E200K, and F198S were purified from cytoplasmic *Escherichia coli* inclusion bodies as described previously (Liemann and Glockshuber, 1999). The mPrP(23–231) D178N variant had methionine at position 129.

For construction and expression of the histidine-to-serine mutants of mPrP(121–231), the previously described phagemid pPrP-CRR was used (Hornemann and Glockshuber, 1996). Site-directed mutagenesis was performed according to the methods of Kunkel (Kunkel, 1985; Kunkel et al., 1991) using uridinylated single-stranded pPrP-CRR from M13K07 phage infection of the *E. coli* strain CJ236 with oligonucleotide primers (5′-CCAATCATTGCCAA AAGATCATGGGTCGACT-3′) for His140Ser, (5′-AAT ATTGACGCAGTCGCTCACGAAGTTGTTCTG-3′) for His177Ser, and (5′-CGTCGTGGTGACCGTCGACTGCTTAATGGT-3′) for His187Ser. Successful mutagenesis was verified by dideoxynucleotide sequencing of the entire PrP genes.

Protein purification

Recombinant mPrP(23–231) and its mutants D178N, E200K, and F198S were purified as described (Liemann and Glockshuber, 1999). After purification, the proteins were dialyzed against distilled water and stored at −20°C after being frozen in liquid nitrogen. The purification of the histidine variants was also performed in accordance with the purification protocol established for the wild-type PrP(121–231) as previously described (Liemann and Glockshuber, 1999). The molecular mass of the purified mutants was confirmed by matrix-assisted ultraviolet (UV) laser desorption/ionization (MALDI) mass spectrometry. Circular dichroism (CD) spectra of

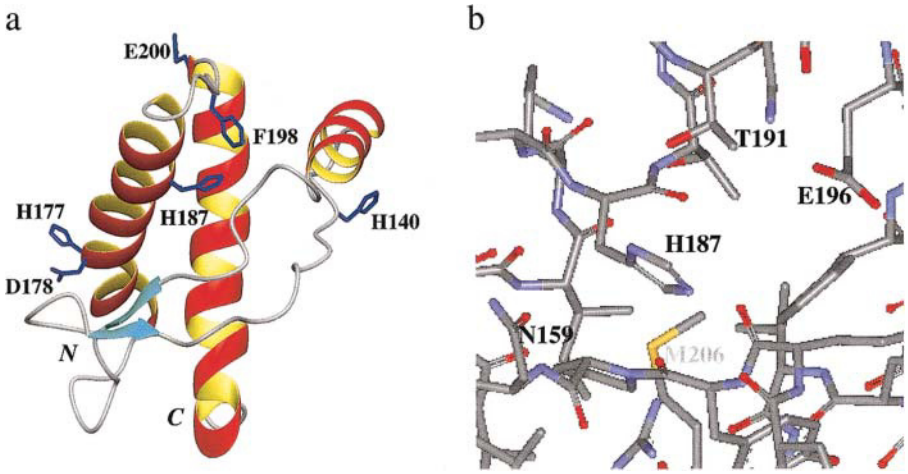

FIGURE 1 (*a*) Three-dimensional structure of the folded C-terminal domain of PrP (PrP(121–231)) showing the location of the amino acid residues mutated in the prion protein variants analyzed in this study. (*b*) Magnification of the histidine 187 environment in the three-dimensional fold of the murine prion protein PrP(121–231).

the mutants confirmed that they folded correctly and had a tertiary structure similar to that of the wild-type protein. The purification yields were in the range of 10–13 mg of pure protein per liter of bacterial culture for the His140Ser and His177Ser mutants, and only 0.2 mg/l of bacterial culture for the His187Ser mutant. The purification of the His187Ser variant from periplasmic inclusion bodies was unsuccessful.

Protein concentrations

Protein concentrations were determined by the specific absorbance of the proteins with the extinction coefficient (ε) = 19,890 $M^{-1} \cdot cm^{-1}$ for mPrP(121–231) and ε = 62,280 $M^{-1} \cdot cm^{-1}$ for mPrP(23–231) (Gill and von Hippel, 1989).

Sample preparation for pH-dependent EPR measurements

These buffers were used (10 mM each): formic acid·NaOH (pH 4.0), sodium cacodylate·HCl (pH 6.0), and 3-(*N*-morpholino)propane-sulfonic acid (MOPS) NaOH (pH 7.4). For the measurements of the Cu(II) binding of wild-type, D178N, E200K, and F198S mPrP(23–231) at the different pH values, 2, 4, and 10 molar equivalents of $CuCl_2$ were added to 0.1 mM of the peptide in 10 mM buffer. For the histidine mutants, one to four molar equivalents of $CuCl_2$ were added to 0.1 mM protein solutions. In most cases, aggregation took place as described (see Results).

Samples for CD spectra

A stock solution of mPrP(23–231) wild type or variants was used to prepare samples of 10 μM PrP(23–231) with or without 0.1 mM $CuCl_2$ in distilled water without buffer or with 10 mM formic acid/NaOH, pH 4.0.

EPR spectroscopy

The EPR spectra were recorded on a Bruker ESP300 spectrometer (microwave frequency, 9.43 GHz) equipped with an ER4131 VT digital temperature-control system, making use of gaseous nitrogen as a coolant. A microwave power of 20 mW, a modulation amplitude of 0.5 mT, and a modulation frequency of 100 kHz were used. All spectra were recorded at a temperature of 120 K. The EPR spectra were simulated using the EasySpin program, a MATLAB toolbox developed for EPR simulations (see http://www.esr.ethz.ch).

CD spectra

Far-UV CD spectra were measured at 22°C on a Jasco 710 CD spectropolarimeter in 0.1-cm quartz cuvettes, accumulated eight times and corrected for the corresponding buffer. Protein samples were centrifuged (5 min, 14,000 rpm, 25°C) before the concentration and CD measurements to remove possible aggregates.

Equilibrium-folding transition studies

The thermodynamic stability of PrP wild type and mutants D178N, F198S, and E200K of both murine PrP(23–231) and PrP(121–231) was analyzed by measuring the CD signal at 222 nm. Urea concentrations of 0–9 M were used as denaturant. The buffer used at pH 7.0 was 45 mM sodium phosphate/NaOH, whereas 50 mM formic acid/NaOH was used at pH 4.0. In addition to these measurements at low ionic strength, another set of experiments was performed at medium ionic strength (135 mM NaCl additionally added to the solutions of mPrP(23–231) and 50 mM NaCl to the solutions of mPrP(121–231)). Medium ionic strength is essential for the formation of the unfolding intermediate of PrP observed at acidic pH (Hornemann and Glockshuber, 1998; Ceregetti et al., in preparation). The protein concentration of both wild-type and mutant proteins was 30 μM for mPrP(23–231) and 20 μM for mPrP(121–231). All transitions were recorded at 22°C.

RESULTS

Figs. 2, 3, and 4 show the X-band EPR spectra of the mPrP(23–231) variants E200K, F198S, and D178N (Fig. 1 *a*) in the presence of four molar equivalents of Cu^{2+} at pH values 4, 6, and 7.4 together with the corresponding spectra of the Cu(II)-bound wild-type protein and the buffer/Cu(II) solutions. The EPR spectra are typical for Cu(II) type-2 complexes (axial **g** matrix, copper hyperfine interaction, $A_{\|} > 400$ MHz). Type-2 complexes are largely square-planar with a possible fifth weak coordination. When the EPR data of different known type-2 copper complexes are plotted in a $g_{\|}$ vs. $A_{\|}$ graph, a correlation can be found between these values and the nature of the four equatorial ligands (Peisach and Blumberg, 1974). Depending on the type of the equatorially coordinating atoms, the $g_{\|}$ and $A_{\|}$ values fall within specific regions. Unfortunately, these regions are overlapping partially, and the $g_{\|}$ and $A_{\|}$ values depend also on the charge of the surrounding ligands and the possible fifth ligand, so that a clear-cut determination of the coordination sphere on the basis of the EPR data alone is not possible. Nevertheless, these parameters can be used to get a first idea about the coordinating atoms.

pH-dependent Cu(II) binding to wild-type mPrP(23–231)

Three Cu(II) coordination types are observed for the wild-type mPrP(23–231) in the pH range of 3–8 (spectra of complexes **1** to **3** in Figs. 2 *b*, 3 *b*, and 4 *b*) (Cereghetti et al., 2001). The EPR spectra are clearly different from those of the Cu(II)/buffer solutions (Figs. 2 *a*, 3 *a*, and 4 *a*). The EPR parameters characterizing complexes **1**–**3** are given in Table 1 (Cereghetti et al., 2001; Van Doorslaer et al., 2001). Complexes **1** (pH 3–6) and **2** (pH 3–8) could be related to the C-terminal part of mPrP(121–231), whereas the third mode of Cu(II) complexation (complex **3**, pH 7–8) is observed in Cu(II)-bound mPrP(121–231), mPrP(58–91), and mPrP (23–231).

At pH values of 4 and 6, up to four molar equivalents of $CuCl_2$ could be added to the full-length protein without significant change in the EPR spectrum other than its intensity (Cereghetti et al., 2001). At higher concentrations of Cu^{2+}, the EPR spectrum was either distorted (appearance of complex **5**, unspecific binding) and/or the EPR spectrum of the Cu(II)-bound buffer was found. The appearance of two contributions to the EPR spectra at these pH values (complexes **1** and **2**) indicated that at least two specific binding sites are present in the C-terminal part of the protein.

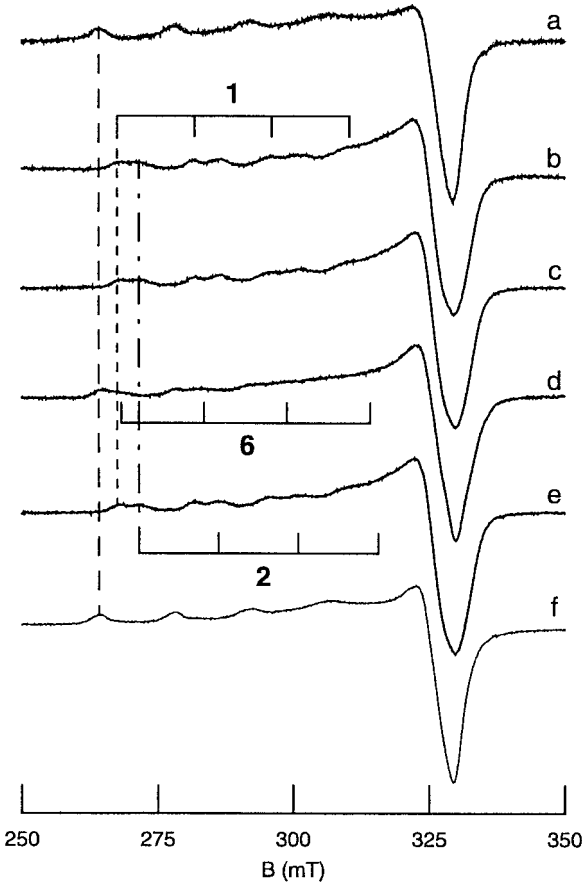

FIGURE 2 X-band EPR spectra at pH 4. (*a*) 0.1 mM $CuCl_2$ in a formic acid·NaOH buffer. (*b*) 0.1 mM mPrP(23–231) with four molar equivalents of $CuCl_2$. (*c*) 0.1 mM of E200K mPrP(23–231) with four molar equivalents of $CuCl_2$. (*d*) 0.1 mM of F198S mPrP(23–231) with four molar equivalents of $CuCl_2$. (*e*) 0.1 mM of D178N mPrP(23–231) with four molar equivalents of $CuCl_2$. (*f*) 0.1 mM of H177S mPrP(121–231) with four molar equivalents of $CuCl_2$.

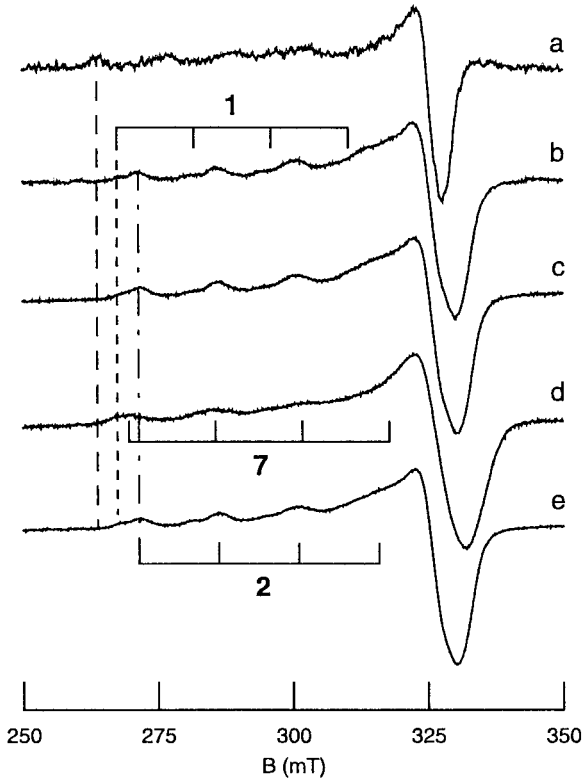

FIGURE 3 X-band EPR spectra at pH 6. (*a*) 0.1 mM $CuCl_2$ in a sodium cacodylate·HCl buffer. (*b*) 0.1 mM mPrP(23–231) with four molar equivalents of $CuCl_2$. (*c*) 0.1 mM of E200K mPrP(23–231) with four molar equivalents of $CuCl_2$. (*d*) 0.1 mM of F198S mPrP(23–231) with four molar equivalents of $CuCl_2$. (*e*) 0.1 mM of D178N mPrP(23–231) with four molar equivalents of $CuCl_2$.

The fact that up to four equivalents of Cu^{2+} could be added without spectral changes can be explained in two ways: either additional EPR-silent binding of Cu(II) occurs (protein or non-protein related) or there are four Cu(II) binding sites in the C-terminal part of the protein with some of them having very similar EPR characteristics. Upon variation of the pH value from 3 to 6, the EPR contribution of complex **1** was found to decrease as the spectrum of complex **2** increased. This indicates that the Cu(II) binding capacity of the two sites is pH dependent, but it is not a clear proof that site **1** evolves into site **2**. The sudden appearance of site **3** at pH 7 whereby site **2** remains present seems to exclude an evolvement of **2** into **3**. At pH values ≥ 7, aquo Cu^{2+} is only sparingly soluble and precipitates as EPR silent $[Cu(OH)_2]_n$. Any excess of Cu^{2+} does not introduce interference in the EPR spectra, but makes a determination of the Cu^{2+}/protein ratio difficult. In the following, the EPR spectra of the different proteins with four molar equivalents of $CuCl_2$ are shown unless aggregation of the protein took place. As mentioned in the experimental part, control experiments were performed with varying molar equivalents.

The CD spectra of wild-type mPrP(23–231) without Cu^{2+} and after addition of 4 and 10 molar equivalents of Cu^{2+} at the different pH values show that the shape of the spectra does not change upon addition of Cu^{2+}, which suggests that no substantial change in protein conformation has occurred (see Supplementary material at http://www.biophysj.org/content/vol84/issue3/).

pH-dependent Cu(II) binding to D178N, E200K, and F198S mPrP(23–231)

The EPR spectra of Cu(II)-bound E200K mPrP(23–231) at pH values of 4 and 6 (Figs. 2 *c* and 3 *c*) are the same as those obtained for the Cu(II)-bound wild-type protein at these pH values (Figs. 2 *b* and 3 *b*). However, at pH 7.4, only complex **2** is visible (Fig. 4, *c* and *c′*), and complex **3** is missing completely (compare with Fig. 4 *b*). The CD spectra of the prion protein variant E200K show that the conformation is insensitive to addition of Cu(II) in agreement with the

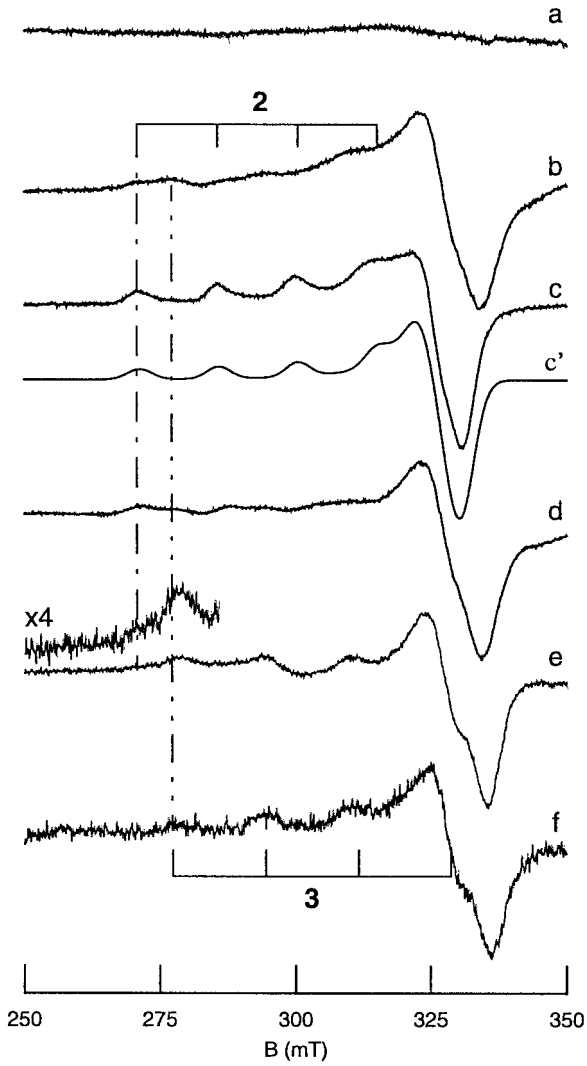

FIGURE 4 X-band EPR spectra at pH 7.4. (*a*) 0.1 mM $CuCl_2$ in a MOPS·NaOH buffer. (*b*) 0.1 mM mPrP(23–231) with four molar equivalents of $CuCl_2$. (*c*) 0.1 mM of E200K mPrP(23–231) with four molar equivalents of $CuCl_2$. (*c′*) Simulation of (*c*) using parameters of complex **2** in Table 1. (*d*) 0.1 mM of F198S mPrP(23–231) with four molar equivalents of $CuCl_2$. (*e*) 0.1 mM of H140S mPrP(121–231) with four molar equivalents of $CuCl_2$. (*f*) 0.1 mM of H187S mPrP(121–231) with one molar equivalent of $CuCl_2$.

behavior of the wild-type protein (see Supplementary material).

At pH 4, the EPR spectrum of Cu(II)-bound F198S differs from the one observed for the Cu(II)-bound mPrP(23–231) (comparison of Fig. 2, *b* and *d*). Apart from the Cu(II)/buffer signal, the spectrum of a new Cu(II) complex is visible (complex **6**). The EPR parameters of complex **6** can be determined after subtraction of spectrum 2 *a* from spectrum 2 *d* (Fig. 2, Table 1). The values agree with a ligation to one nitrogen and three oxygens (1N3O), two nitrogens and two oxygens (2N2O), or three nitrogens and one oxygen (3N1O) (Peisach and Blumberg, 1974). These values clearly deviate from those of complexes **1** and **2**. At pH 6, only one Cu(II) complex is observed for the F198S mutant (Fig. 3 *d*; Table 1, complex **7**). The EPR parameters are again in agreement with a 1N3O, 2N2O, or 3N1O coordination (Peisach and Blumberg, 1974). At pH 7.4, complex **3** is visible in the EPR spectrum of Cu(II)-bound F198S (Fig. 4 *d*). Furthermore, a second component similar to complex **2** is visible in the spectrum. However, the relative ratio of the contributions of complexes **2** and **3** is different from the one observed for the wild-type protein. Because complex **2** was not observed for the mutant at pH values of 4 and 6, it is possible that the copper coordination site **2** observed for F198S at pH 7.4 is similar but not exactly the same as the one observed in the wild-type protein (hence the question mark in Table 1). Furthermore, the CD spectra show that the Cu(II) binding has no substantial influence on the protein conformation (see Supplementary material).

The full-length D178N mutant of the murine prion protein is found to aggregate at pH 7.4 both in the presence and absence of copper. At pH values of 4 and 6, the EPR spectra and CD spectra of Cu(II)-bound D178N are the same as those of the Cu(II)-coordinated wild-type protein (Figs. 2 *e* and 3 *e*, and Supplementary material).

Stability of H177S, H187S, and H140S mPrP(121–231) with and without copper

Comparison of the CD spectra of 10 μM of the histidine mutants (Fig. 1 *a*) and of the wild-type mPrP(121–231) at pH 4 (not shown) indicates that the variants are not properly folded at this pH value in 10 mM formic acid·NaOH buffer. Accordingly, no Cu(II) binding to the protein was found by EPR. The EPR spectra of all histidine variants after addition of Cu^{2+} is equal to that of the Cu(II)-buffer complex as is shown in Fig. 2 *f* for the H177S mutant (compare with Fig. 2 *a*). This indicates that the protein conformation is essential for the binding of copper to the prion protein at this pH.

Already in the presence of one molar equivalent of Cu^{2+}, strong aggregation of all the histidine variants (0.1 mM) occurred at pH 6 in 10 mM cacodylate·HCl buffer.

At pH 7.4, the H140S mutant is soluble even upon addition of four molar equivalents of $CuCl_2$. The EPR spectrum shows a clear contribution of complex **3** (Fig. 4 *e*). Furthermore, a small contribution of complex **2** is found (see amplification in low-field area of spectrum in Fig. 4 *e*). The H177S mutant aggregates immediately upon addition of even one molar equivalent of Cu^{2+}. Although the H187S variant also aggregates in the presence of one molar equivalent of Cu^{2+}, the aggregation process is found to be much slower than for the H177S mutant. The EPR spectrum of the Cu(II)-bound H187S obtained by freezing the sample after addition of $CuCl_2$ and before visual protein aggregation had set in shows the characteristic features of complex **3** (Fig. 4 *f*). We cannot exclude, however, that aggregation was already in process and the data should be interpreted cautiously.

TABLE 1 EPR parameters of the type-2 Cu(II) complexes observed in copper-containing full-length mPrP(23–231) and its mutants F198S, D178N, and E200K and in the mPrP(121–231) mutated forms H140S and H187S

Complex	$g_\perp$ (±0.005)	$g_\parallel$ (±0.005)	$A_\perp$ (±10 MHz)	$A_\parallel$ (±10 MHz)	Observed in	pH
1	2.068	2.332	12	452	mPrP(121–231)*	3–6
					mPrP(23–231)*	4, 6
					D178N†	4, 6
					E200K†	
2	2.068	2.295	20	457	mPrP(121–231)*	3–8
					mPrP(23–231)*	4, 6
					D178N†	4, 6, 7.4
					E200K†	7.4
					F198S (?)†	7.4
					H140S†	
3	2.055	2.230	50	495	mPrP(121–231)*	7–8
					mPrP(58–91)*	7.4
					mPrP(23–231)*	7.4
					F198S†	7.4
					H140S†	
					H187S†	
4	2.055	2.270	50	520	mPrP(58–91)*	6, 7.4
5	2.114		Not resolved		mPrP(23–231)* (>4 equiv. Cu^{2+})	4, 6
6	2.058	2.320	35	480	F198S†	4
7	2.061	2.295	10	510	F198S†	6

Hyperfine data are given for ^{63}Cu.
*Data from Cereghetti et al., 2001.
†Data obtained in present study.

Equilibrium-folding transition experiments

For the recombinant mPrP(121–231) wild type and mutants D178N, F198S, and E200K, measurements at pH 4.0 in the presence and absence of 50 mM NaCl were done and compared to the previous results found for the same proteins at pH 7 in the absence of salt (Liemann and Glockshuber, 1999). The thermodynamic parameters are reported in Table 2. The thermodynamic stability of mPrP(23–231) and mutants D178N, F198S, and E200K was analyzed at pH 4.0 (Fig. 5) and at pH 7.0 in the absence and presence of 135 mM NaCl. The corresponding thermodynamic parameters are reported in Table 3.

The folding was found to be a reversible process for all the mutants at both pH values. In general, the mutant E200K is found to be almost as stable as the wild-type protein under all conditions tested. The mutants D178N and F198S, which were previously found to show a reduced intrinsic stability at pH 7.0 for the mPrP(121–231) variants (Liemann and Glockshuber, 1999), are also less stable than the wild-type protein at both pH 7.0 and 4.0 in the case of the full-length mutants. The thermodynamic stability of the C-terminal domain alone, mPrP(121–231), and the full-length protein mPrP(23–231) for the wild type as well as for the mutants is approximately the same.

A major change in folding and stability of all the proteins investigated is observed by increasing the ionic strength at acidic pH (Fig. 5, *b* and *c*), and the formation of the folding intermediate described previously (Hornemann and Glockshuber, 1998; Cereghetti et al., in preparation) is also found for the mPrP variants under study. No determination of ΔG^o can be obtained, because of the concentration dependence of the formation of the PrP^{Sc}-like folding intermediate and the fact that the oligomeric state of this intermediate is unknown.

In summary, as shown in Tables 2 and 3, even at acidic pH values, the thermodynamic stability data indicate that the mutants investigated are not uniformly destabilized with respect to the wild type.

Interestingly, the mPrP(23–231) variants D178N and F198S are found to aggregate at pH 7.0 where no unfolding intermediate is found for the wild-type protein. This aggregation is most likely unspecific and is attributable to the decreased stability of the variants with respect to the wild type at neutral pH. On the other side, no aggregation of these two mutant proteins is observed at pH 4 and at an ionic strength >100 mM, i.e., under the conditions where the unfolding intermediate is formed in the wild-type PrP(23–231). The soluble intermediate may therefore be a precursor of PrP^{Sc} analogous to the one formed during unfolding of wild-type mPrP(23–231). The intermediate formation occurs at pH 4.0 and in the presence of 165 mM ionic strength at the same urea concentration for all the mutants as was observed for the wild type. Indeed, at 3.5 M urea, an intermediate state with CD spectra identical to the one of the wild type and reminiscent of PrP^{Sc} is found. It seems therefore that the oligomeric unfolding intermediate is also populated in the case of the variants related to prion diseases. No intermediate formation is found at ionic strengths <50 mM.

TABLE 2 Thermodynamic stability of mPrP(121–231) wild-type and variants at pH 4 and 7 without NaCl

	ΔG° (kJ/mol)	ΔΔG° (kJ/mol)	Cooperativity of folding (kJ/mol M)	$[urea]_{1/2}$ (M)
pH = 4.0;				
Ionic strength = 30 mM*				
Wild type	−16.9 ± 1.4		5.0 ± 0.4	3.4
E200K	−17.6 ± 1.0	0.7 ± 2.4	5.5 ± 0.3	3.2
F198S	−12.2 ± 1.1	4.7 ± 2.5	4.3 ± 0.3	2.8
D178N	−12.0 ± 0.9	4.9 ± 2.3	4.5 ± 0.3	2.7
pH = 7.0†;				
Ionic strength = 80 mM*				
Wild type	−29.7 ± 1.0		4.8 ± 0.2	6.2
E200K	−29.1 ± 1.5	0.6 ± 2.4	4.9 ± 0.3	5.9
F198S	−19.4 ± 0.8	10.3 ± 1.7	4.4 ± 0.2	4.5
D178N	−22.5 ± 0.8	7.2 ± 1.7	4.7 ± 0.1	4.8

*In the absence of NaCl.
†Data from Liemann and Glockshuber, 1999.

TABLE 3 Thermodynamic stability of mPrP(23–231) wild type and variants at pH 7 and 4 (22°C) at different ionic strengths

	ΔG° (kJ/mol)	ΔΔG° (kJ/mol)	Cooperativity of folding (kJ/mol M)	$[urea]_{1/2}$ (M)
pH = 4.0				
Ionic strength = 30 mM				
[NaCl] = 0				
Wild type	−17.3 ± 0.6		4.9 ± 0.2	3.53
E200K	−16.4 ± 0.6	0.9 ± 1.2	5.2 ± 0.2	3.15
F198S	−9.5 ± 0.2	7.8 ± 0.8	3.8 ± 0.1	2.50
D178N	−9.4 ± 0.2	7.9 ± 0.8	4.0 ± 0.1	2.35
pH = 7.0				
Ionic strength = 80 mM				
[NaCl] = 0				
Wild type	−25.6 ± 1.6		4.2 ± 0.3	6.09
E200K	−25.3 ± 1.7	0.3 ± 3.3	4.4 ± 0.3	5.75
F198S	*			
D178N	*			
pH = 7.0				
Ionic strength = 215 mM				
[NaCl] = 135 mM				
Wild type	−26.4 ± 1.7		4.4 ± 0.3	6.00
E200K	−27.4 ± 1.5	−1 ± 3.2	4.9 ± 0.3	5.59
F198S	*			
D178N	*			

*Could not be evaluated quantitatively due to partial aggregation during the transition.

DISCUSSION

General remarks

Copper binding to PrPs is a highly debated matter in PrP research. Apart from the extensive discussions on the possible involvement of copper in the in vivo function of PrP^C or in the interconversion between PrP^C and PrP^{Sc} (Brown et al., 1997; Pauly and Harris, 1998; Wadsworth et al., 1999; Thompson et al., 2000; Waggoner et al., 2000), there is still no general consensus on the interpretation of biophysical experiments concerning the in vitro copper binding of prion proteins.

All physicochemical experiments performed up to now confirm that Cu(II) can bind at pH values >5 to the octarepeat peptide part of the N-terminal region of PrP^C (Hornshaw, McDermott, and Candy, 1995; Hornshaw et al., 1995; Stöckel et al., 1998; Viles et al., 1999; Miura et al., 1999; Whittal et al., 2000; Aronoff-Spencer et al., 2000; Cereghetti et al., 2001; Jackson et al., 2001; Kramer et al., 2001; Burns et al., 2002; Qin et al., 2002). Raman and EPR measurements show that different pH-dependent copper-binding modes occur (Miura et al., 1999; Aronoff-Spencer et al., 2000; Cereghetti et al., 2001). However, there is still no agreement on the number of Cu(II) ions that are bound nor on the binding affinity (K_d values of 10^{-14} M up to several micromoles are reported;

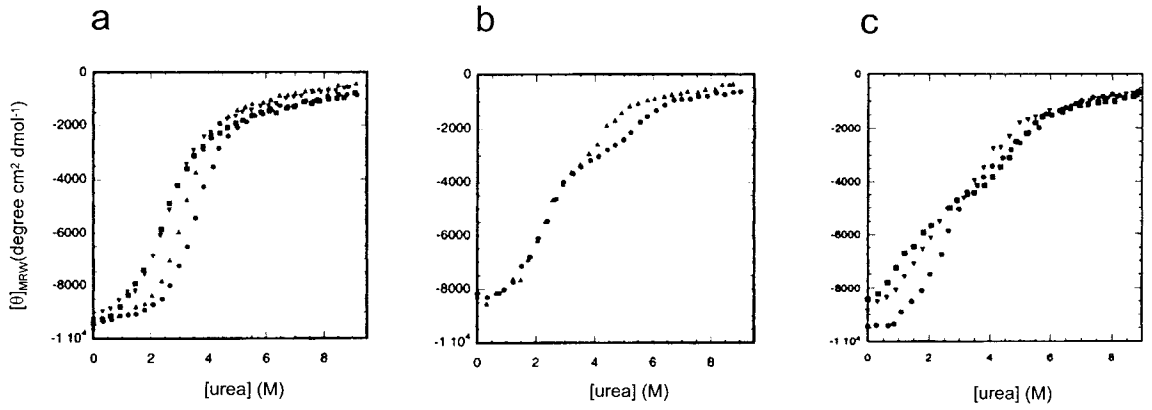

FIGURE 5 (*a*) Unfolding transitions of mPrP(23–231) at pH 4 and at an ionic strength of 30 mM (22°C). Protein concentration, 28 μM. mPrP(23–231) wild type (●), variant E200K (▲), variant F198S (▼), and variant D178N (■). (*b*) Unfolding transitions of mPrP (23–231) at pH 4 and at an ionic strength of 165 mM (22°C). Protein concentration, 28 μM. mPrP(23–231) wild type (●) and variant E200K (▲). (*c*) Unfolding transitions of mPrP (23–231) at pH 4 and at an ionic strength of 165 mM (22°C). 14.5 μM mPrP(23–231) wild type (●), 20 μM mPrP(23–231) F198S (▼), and 20 μM mPrP(23–231) D178N (■).

Hornshaw, McDermott, and Candy, 1995; Hornshaw et al., 1995; Stöckel et al., 1998; Viles et al., 1999; Miura et al., 1999; Whittal et al., 2000; Aronoff-Spencer et al., 2000; Jackson et al., 2001; Kramer et al., 2001). These discrepancies are probably due to the different experimental conditions used (such as the pH values and the model of PrP fragment analyzed) as well as buffer artifacts as previously highlighted (Cereghetti et al., 2001).

More recently, Cu(II) binding was observed also upstream of the octapeptide repeat region and in the C-terminal structured region (Cereghetti et al., 2001; Jackson et al., 2001; Kramer et al., 2001; Hasnain et al., 2001; Qin et al., 2002). Here we analyze by EPR the Cu(II) binding to some point-mutation variants of the recombinant murine PrP(23–231) related to inherited TSE forms in humans (Fig. 1 *a*). The fact that the EPR spectra of the Cu(II)-bound prion proteins were found to change for different point mutations in the C-terminal part proves that the already previously observed Cu(II) complexes in the EPR spectra of the C-terminal domain PrP(121–231) can be related to specific binding sites in the protein. Furthermore, the EPR spectra of the improperly folded mPrP(121–231) H140S, H177S, and H187S mutants with added Cu(II) at pH 4 (Fig. 2 *f*) lacked the characteristic features of the Cu(II)-bound mPrP(121–231) and mPrP(23–231), which proves that a correct protein folding is absolutely essential for Cu(II) binding at low pH values.

Cu(II) binding to the recombinant mPrP(23–231) wild type

In our earlier EPR results (Cereghetti et al., 2001; Van Doorslaer et al., 2001), we showed that both the full-length prion protein mPrP(23–231) and the C-terminal fragment mPrP(121–231) have similar binding of Cu^{2+} ions from pH values $\geq$3, whereas the N-terminal part, mPrP(58–91), only binds at pH values >5 (Table 1). Furthermore, the Cu(II) coordination type observed for mPrP(58–91) at pH 6 (complex **4**, Table 1) differs from the binding types observed for mPrP(23–231) and mPrP(121–231). Based on this evidence, complexes **1** and **2** observed in the EPR spectra of the Cu(II)-bound, full-length, wild-type prion protein (Figs. 2 *b*, 3 *b*, and 4 *b*) could be assigned to binding sites in the structured C-terminal part of the protein. Pulse EPR and electron-nuclear double resonance (ENDOR) investigations showed that one of the binding ligands in complex **2** is histidine and that no nitrogen is directly coordinated to the copper in complex **1** (Van Doorslaer et al., 2001). Complex **3**-type contributions were found in the EPR spectra of Cu(II)-containing mPrP(23–231), mPrP(121–231), and mPrP(58–91) at pH values $\geq$7 (Fig. 4 *b*). In view of the recent observation of a Cu(II) binding site outside the octarepeat region involving His-96 and His-111 (Jackson et al., 2001), our findings indicate that at least three binding sites with similar EPR parameters are present in the full-length protein. It should be noted that due to the broad line width of the EPR spectra, small differences in the EPR parameters cannot be detected. The sites are therefore most likely to be similar but not identical. Pulse ENDOR measurements indicated that more than one nitrogen atom is directly bound to the copper ion, and similarities between the *g* and copper hyperfine values of complex **3** and those of known 1:2 Cu-peptide complexes in alkaline solutions (Szabó-Plánka et al., 1989) suggested that deprotonated backbone nitrogens can be involved in the binding. Due to the fact that the same type of Cu(II) binding was found in mPrP(23–231), mPrP(121–231), and mPrP(58–91), involvement of the N-terminal amino group could not be ruled out (Cereghetti et al., 2001). We therefore plan to check this possibility by putting an acetyl group on the N-terminus. Furthermore, upon addition of more than four molar equivalents of Cu^{2+} to mPrP(23–231) at pH 4 or 6, a broad signal was observed (complex **5**, Table 1) which could be ascribed to unspecific binding of the Cu(II) ions to the protein (Cereghetti et al., 2001).

The present CD measurements on the recombinant mPrP(23–231) wild type in the absence and presence of up to 10 molar equivalents of Cu(II) at pH values of 4.0, 6.0, and 7.4 show that no substantial conformational change takes place upon addition of copper (see Supplementary material), which indicates that the N-terminal region still remains unstructured also in the presence of bound Cu(II). This is contrary to earlier speculations that the N-terminal region becomes structured upon binding, perhaps assuming an α-helixlike secondary structure (Viles et al., 1999; Stöckel et al., 1998; Wong and Clive et al., 2000; Wong and Venien-Bryan et al., 2000). This agrees, however, with recent molecular modeling computations which showed that although metal binding to the repeat region would render this region more compact than in the apoprotein, it would not lock it into any single rigidly defined conformation (Jackson et al., 2001).

Which histidine is involved in complex 2?

To test directly which histidine of the C-terminal part is involved in the copper binding site **2**, the mPrP(121–231) mutants H177S, H187S, and H140S were expressed (Fig. 1 *a*). At pH 4, none of the peptides were properly folded even in the absence of copper as shown by CD spectroscopy, and at pH 6, all the mutants aggregated upon addition of copper. Because H177S mPrP(121–231) aggregated also upon addition of copper at pH 7.4, no direct verification of the involvement of His-177 could be done. However, the EPR spectra of the Cu(II)-bound D178N and of the Cu(II)-bound wild-type prion protein at pH values of 4 and 6 are identical (compare Figs. 2 *b* and 3 *b* and 2 *e* and 3 *e*). This indicates that His-177 is not involved in complex **2**. The change from aspartic acid to asparagine will modify the environment of His-177 considerably (removal of salt bridge between Asp-178 and Arg-164 (Riek et al., 1997; Zuegg and Gready,

1999)), which should lead to a change in the EPR spectrum **2**. Furthermore, recent MALDI-time-of-flight experiments on Cu(II)-bound human PrP failed to detect involvement of H176 (Qin et al., 2002), which confirms our present finding.

At pH 7.4, the mPrP(121–231) H140S variant did not aggregate upon addition of one tofour molar equivalents of Cu^{2+}. Besides complex **3**, the EPR spectrum of complex **2** is visible (Fig. 4 *e*). The observation of complex **2** seems to rule out the participation of His-140 in this binding site. The intensity ratio of spectrum **2** to **3** is smaller for the mPrP(121–231) H140S variant than for the full-length mPrP(23–231) wild type, but is similar to the one found for the wild-type mPrP(121–231) fragment (Cereghetti et al., 2001). This may indicate that the copper affinity at site **2** decreases when the mPrP(23–120) part is missing.

Only the possible involvement of His-187 in complex **2** remains (Fig. 1 *b*). Indeed, no contribution of complex **2** is found in the EPR spectrum of mPrP(121–231) H187S after addition of one molar equivalent of copper at pH 7.4 (Fig. 4 *f*). Owing to the slow aggregation of mPrP(121–231) H187S upon addition of copper, this observation should be interpreted with caution.

Additional indications for a His-187-copper bond can be found in the observation that the EPR spectra of the Cu(II)-bound mPrP(121–231) F198S variant are different from those of the Cu(II)-bound mPrP(23–231) wild type. Previous studies showed that the replacement F198S strongly destabilizes the prion protein. The hydrophobic phenylalanine residue buried in the hydrophobic core of the protein in the region between helices 2 and 3 is replaced by the nonhydrophobic residue serin and possibly induces local structural rearrangements within the protein scaffold (Riek et al., 1998; Liemann and Glockshuber, 1999). These rearrangements within the C-terminal domain might dislocate the Cu(II) ligands within the region where complexes **1** and **2** are formed. If His-187 is responsible for Cu(II) binding leading to the features of complex **2**, the fact that this residue comes in the near vicinity of the site of mutation at position 198 in the three-dimensional fold could explain the absence of complex **2** due to an altered environment of His-187 (Fig. 1). Histidine residues 177 and 140 are at a larger distance from residue 198 (Fig. 1 *a*). The fact that new types of Cu(II) complexation are observed at these pH values (complexes **6** and **7**), for which the EPR parameters support nitrogen involvement in the direct ligation of the copper, would indicate that His-187 is still binding to the copper ion but that the binding environment has changed. Different potential additional ligands for Cu(II) are present in the vicinity of His-187 (Fig. 1 *b*). The new binding environment has also become strongly pH dependent (change of complex **6** to **7**). At pH 7.4, a contribution similar to that of complex **2** is observed for the Cu(II)-bound mPrP(23–231) F198S variant. This might be explained in two ways. Because the observed complexes in the wild type and the mutant at pH 7.4 are only similar within the error of the EPR measurement, they may have slightly different local symmetries. On the other hand, it is possible that the same complex as in the wild-type protein is observed, but that due to the mutation and consequent change in the local structure, its formation is only possible at a higher pH value. This might be the case if in the wild-type protein the different copper ligands are forced together by sterical effects that facilitate copper ligation, whereas in the mutant, the ligands are no longer at a favorable position and a higher pH value (i.e., deprotonation of the ligands) is needed to enable the complex formation.

Finally, at pH 4 and 6, the spectra of the Cu(II)-bound E200K mutant look the same as those of the Cu(II)-bound wild-type protein (Figs. 2 *c* and 3 *c*), and complex **2** is found in the EPR spectrum of the Cu(II)-coordinated mutant at pH 7.4. Because residue 200 is not in the vicinity of His-140, His-177, or His-187 (Fig. 1 *a*), this does not contradict our earlier pulse-EPR results that one of these histidines is involved in complex **2** (Van Doorslaer et al., 2001).

How to interpret complex 3?

As mentioned before, complex **3**-type binding was found in mPrP(23–231), mPrP(121–231), and mPrP(58–91) (Cereghetti et al., 2001). Because of the resemblance between the EPR parameters of complex **3** and those of Cu(II)-peptide complexes that involve coordination to deprotonated backbone nitrogens and end-amino groups, it was not clear to which part of the full-length protein the copper-binding site **3** could be ascribed, and the possibility of spurious binding to one or all of the studied peptides had to be taken into account. Interestingly, we now observe that complex **3** is not formed in the Cu(II)-bound mPrP(23–231) E200K variant (Fig. 4 *c*), whereas it is found for the mPrP(121–231) H140S and H187S and the mPrP(23–231) F198S variants (Fig. 4, *e*, *f*, and *d*, resp.). The E200K mutation is situated at the beginning of the third α-helix of the C-terminal part and is located at the surface of the protein (Riek et al., 1998). The disappearance of the contribution of complex **3** in the spectrum of Cu(II)-bound E200K links the formation of this complex in the full-length protein to a binding site around residue 200 in the C-terminal part. It seems to exclude that this complex is the same as is observed in Cu(II)-bound mPrP(58–91), because this region is not affected by the point mutation. This also agrees with the fact that for the F198S variant, the spectral intensity of **2** is larger than that of **3**, whereas this is the inverse in mPrP(23–231) wild type. Residue 198 is near to residue 200 (Fig. 1 *a*) and the F198S mutation might already hinder formation of complex **3** due to a change in the hydrophobicity of the site.

The observation that at pH 7.4, only complex **3** is visible in Cu(II)-bound H187S mPrP(121–231) seems to corroborate the earlier assumption that the two C-terminal sites **2** and **3** do not evolve into each other. Indeed, the latter finding would indicate that the histidine ligand in

complex **2** no longer coordinates when the site evolves into complex **3**, which seems very unlikely, because histidine binding should be favored at higher pH values.

Where is complex 1 located?

Our earlier results (Cereghetti et al., 2001; Van Doorslaer et al., 2001) showed that complex **1** is only found at pH values $\leq$6, and that no nitrogens are involved in the direct binding to the copper. This complex is found for mPrP(23–231) D178N and for E200K, which indicates that this copper-binding site is neither centered around residues 178 and 200 nor around residue 164 (Asp-178 forms a hydrogen bond to Arg-164 in the wild-type prion protein (Riek et al., 1998)) nor around residue 128 (Tyr-128—Asp-178 hydrogen bond (Riek et al., 1998)). Complex **1** is not formed in Cu(II)-bound F198S. This does not necessarily mean that the binding site is centered around residue 198. The replacement of Phe-198 in the hydrophobic core by Ser is a nonconservative mutation. Without follow-up structural changes, this mutation would lead to an empty cavity that could accommodate 2 or 3 water molecules. In the wild-type protein, the aromatic ring is surrounded by numerous hydrogen-bonded polar side chains, and replacement by serine will surely modify these polar interactions (Riek et al., 1998). One of these interactions is the hydrogen bond between Arg-156 and Glu-196 (Riek et al., 1998; Zuegg and Gready, 1999). Interestingly, the NMR study of Cu(II)-bound mPrP(91–231) at pH 6 showed a copper-binding site centered around residues 135 and 155 upon titration above stoichiometry (Jackson et al., 2001). Because complex **1** is still formed at this pH, it is tempting to relate these observations. In addition, the posttranslational glycosylation at asparagine residues 181 and 197 could influence copper binding as well.

Finally, the present study still does not allow us to determine whether site **1** evolves into site **2** upon pH increase. The fact that the EPR spectra of complexes **1** and **2** remain unchanged or are altered simultaneously upon point mutations seems to support a relation between the two complexes but cannot prove it beyond doubt.

Biological relevance

Relating the in vitro analytical data on Cu(II) binding to prion proteins to the in vivo binding is difficult, because PrP^C is a membrane protein. It is attached to the cell surface via a glycosyl phosphatidylinositol anchor and is supposed to have a high level of lateral mobility within the membrane. This makes determination of the local protein concentration difficult. Furthermore, the prion protein might interact with another protein at the membrane that stabilizes PrP^C-Cu(II) complexes. Nevertheless, some of our present observations are interesting in view of the biological function.

On the one hand, the role played by the point mutations in facilitating the process of disease formation is still not clarified. Destabilization of the three-dimensional fold of PrP^C for the mutant proteins was hypothesized as the cause of the increased propensity of the mutants to convert to the infectious isoform PrP^{Sc} (Prusiner, 1997; Cohen et al., 1994; Huang et al., 1994). Studies of the thermodynamic stability of the corresponding variants of murine PrP(121–231) at pH 7.0 (Liemann and Glockshuber, 1999) and of some variants of human PrP(90–231) at pH 3.6–7.2 (Swietnicki et al., 1998) excluded this possibility as a general explanation for spontaneous prion formation in inherited TSEs as they showed that not all of the mutants were apparently destabilized by the point mutation. The observed urea-induced equilibrium-folding transitions at pH values of 4.0 and 7.0 of the full-length mPrP variants D178N, F198S, and E200K allowed us to confirm that also for the full-length protein and even under acidic pH conditions, no uniform destabilization of the mutated proteins is observed (Tables 2 and 3). Acidic conditions are found in the endocytic pathway, where the PrP^C to PrP^{Sc} conversion is supposed to take place (Lee et al., 1996; Hornemann and Glockshuber, 1998). Furthermore, the dependence of intermediate formation on the protein concentration appears to vary for the different protein variants. The D178N variant of PrP(23–231) already presents a strongly populated intermediate at a 20 μM protein concentration (Fig. 5 *c*), whereas the E200K variant shows minimal intermediate population at 3.5 M urea even at a protein concentration of 30 μM (Fig. 5 *b*). This observation prompts us to assume that the point mutation may facilitate or hinder oligomerization of the protein on the way to PrP^{Sc} formation. This may perhaps influence the interaction with other factors involved in normal PrP metabolism if temporary oligomerization plays a functional role in the cell.

On the other hand, the finding that PrP is able to bind Cu(II) in vivo (Brown et al., 1997) prompted the suggestion that Cu(II) is involved in the biological function of the prion protein, although the subject is still highly controversial. The recent findings that the C-terminal part of the prion protein can bind Cu(II) (Thompson et al., 2000; Kramer et al., 2001; Cereghetti et al., 2001; Van Doorslaer et al., 2001; Jackson et al., 2001) are important in view of the fact that the known pathogenic mutations take place in the PrP(102–238) part of the protein (Collinge, 2001) and in view of the observation that metal-bound active centers are usually located in the structured region of a protein, where the coordination partners are stabilized by the protein scaffold. The CD measurements show that copper induces no substantial conformational changes in the recombinant mPrP(23–231) wild type nor in its variants (see Supplementary material), so that it seems unlikely that the N-terminal part gains a fixed structure as suggested earlier (Viles et al., 1999; Stöckel et al., 1998; Wong and Clive et al., 2000; Wong and Venien-Bryan et al., 2000).

In vitro experiments on the Cu(II) binding of prion proteins are evidently important to understand the in vivo Cu(II)-related behavior, but the in vitro Cu(II) binding of the protein can also be seen as a sensor for the local protein structure. The differences in the in vitro Cu(II) binding between the mPrP(23–231) variants D178N, F198S, and E200K, which correspond to three different phenotypes of inherited prion diseases in humans, and the wild-type protein as detected by EPR are necessarily related to changes in the local structure of the protein.

Our present results strongly suggest that His-187 is involved in the binding site **2** found in mPrP(23–231) wild type. This is interesting in view of a number of earlier observations.

First of all, PrP(178–193) was found to promote Cu(II)-induced lipid peroxidation and cytotoxicity in primary neuronal cultures, whereas PrP(144–154) and PrP(198–218) had no effect on the Cu(II) toxicity (Thompson et al., 2000). There was no increase in toxicity induced by PrP(178–193) in cultures treated with Fe(II) or hydrogen peroxide, indicating a preferential modulatory effect on Cu(II) toxicity by PrP(178–193). His-187 is centered well within this protein segment.

Recently, an inherited prion-related encephalopathy resembling the GSS disease was discovered and associated with the H187R mutation (Cervenakova et al., 1999; Butefisch et al., 2000). This is the only known histidine mutation associated with familial encephalopathy, and for this mutation, complex **2** formation would clearly be impossible. It is interesting to point out that also the F198S mutant associated with the GSS disease did not form complex **2** upon addition of copper at a lower pH. Even if the copper-binding capacity of the prion protein has no biological meaning, Cu(II) can be seen as a sensor to detect local changes in the protein upon mutations. The copper-binding behavior of the two mutants H187R and F198S related with the same phenotype of prion diseases in humans shows that both mutations cause local structural changes around residue 187. The latter could also explain why Forloni et al. (1999) found no marked difference in the neurotoxicity of the PrP(195–213) wild type and F198S mutated segment. This segment is missing the region around residue 187. A comparative NMR analysis of H187R, F198S, and other mutants associated with the GSS phenotype would therefore be very interesting.

The fact that the mutation E200K causes the inherited form of CJD is still puzzling. The NMR structure shows that the mutation is located on the protein surface (Fig. 1 *a*) and predicts that even the change in overall charge of the protein should not have a major impact on the global structure. Indeed, the thermodynamic stability of the E200K variant is similar to that of the wild-type protein (Table 3). Interestingly, our present study shows that the mPrP(23–231) E200K mutant shows a marked difference in its copper binding at pH 7.4 (complex **3** is missing from the EPR spectrum). In this view, the recent analysis of the metal occupancy both of the brains and of the PrP of patients with sporadic CJD is interesting (Wong et al., 2001). The authors found an increase of manganese and, to a lesser extent, of zinc accompanied by significant reduction of copper bound to the purified PrP of all sporadic CJD variants. The latter reduction gives an interesting link to the observation that at neutral pH, complex **3** is missing from the EPR spectra of Cu(II)-bound E200K, the point mutation leading to the inherent form of CJD.

The mPrP(23–231) D178N variant shows similar copper-binding behavior at pHvalues ≤ 6 as the wild-type prion protein, but it is found to aggregate at pH 7.4 both in the absence and presence of copper. Reduced stability of D178N peptide segments in the absence of copper has also been reported earlier. mPrP(121–231) D178N could not be obtained in soluble form in the periplasm and showed reduced thermodynamic stability (Liemann and Glockshuber, 1999). Turbidimetric analyses indicated an increase in the aggregating capacity of PrP(169–185) D178N in comparison to the wild-type peptide segment (Forloni et al., 1999). The human PrP(90–231) D178N mutant was found to aggregate in the inclusion bodies of *E. coli* (Swietnicki et al., 1998). This all agrees with the NMR prediction and molecular dynamics simulations that the D178N mutation removes the salt bridge between Asp-178—Arg-164, which will thermodynamically destabilize the mutant (Riek et al., 1998; Zuegg and Gready, 1999). Furthermore, Asp-178 is in close proximity to the S—S bridge. A mutation in this region may potentially interfere with the disulfide-bridge formation. Our findings show that in vitro, the main difference between the wild type and the mutant lies in the self-aggregation at pH 7.4 and not in the copper-binding behavior.

In conclusion, this study shows that protein destabilization alone cannot account for the spontaneous pathogenic activity of all the known mutations. The change observed in the in vitro Cu(II) binding behavior of the mPrP(23–231) variants E200K and F198S opens the possibility that protein destabilization and altered Cu(II) binding behaviors are responsible for the pathological effects, and that the interplay between them might be related to the phenotype of the different prion diseases.

This study thus opens the way to future experiments to unravel further the Cu(II) binding sites and their biological meaning in prion proteins. The involvement of the N-terminal amino group in complex **3**-type binding sites should be tested by putting an acetyl group on the N-terminus. Furthermore, the Cu(II) binding site involving His-96 and His-111 (Jackson et al., 2001) should be addressed in a future study by the analysis of the EPR spectra of full-length mPrP and of its specific His-96/His-111 mutants. Finally, to determine a possible relationship between specific pathological effects and altered Cu(II) binding and/or protein destabilization, a larger number of prion mutants should be studied.

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